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Inhibition of reactive oxygen species and pre-neoplastic lesions by quercetin through an antioxidant defense mechanism

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Abstract

There is a correlation between oxidative stress generated by diethylnitrosamine (DEN) metabolism and liver cancer development. Quercetin is a flavonoid with anti-carcinogenic and antioxidant properties. This study demonstrates the mechanism of action for the chemopreventive effect of quercetin. A 10 mg/kg dose of quercetin produced drastic effect, when it is administrated 2 h before DEN; at 24 days post-DEN, a 70.3% and 66.2% decrease in total area and number of preneoplastic lesions were observed, respectively. At 12 h post-DEN, quercetin inhibited levels of lipid peroxidation by 40%. Quercetin increased the levels of both GSH and of total glutathione, it increased the GSH/GSSG index and it caused a rapid and simultaneous elevation in the activities of superoxide dismutase, glutathione peroxidase and catalase. In conclusion, the quercetin mechanism of action is due to promote the enzymatic and non-enzymatic antioxidant defense system during the initiation of hepatocarcinogenesis.

Keywords: *Oxidative stress, hepatocarcinogenesis, antioxidant defense system, chemoprevention, quercetin*

Abbreviations: *HNE, 4-hydroxy-2-nonenal; 2-AAF, 2-acetylaminofluorene; CAT, catalase; DEN, diethylnitrosamine; GGT, gamma-glutamyl-transpeptidase; GSSG, oxidized glutathione; GSH, reduced glutathione; GPx, glutathione peroxidase; GST, glutathione S-transferase; MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase.*

Introduction

Reactive oxygen species (ROS) are molecules with high chemical reactivity. They include free radicals such as hydroxyl ($^{\bullet}OH$) and superoxide ($O_2^{\bullet}$) and non-radicals such as hydrogen peroxide (H_2O_2) and singlet oxygen (O_2) [1,2]. ROS are unstable and react rapidly with other free radicals and biomolecules in chain reactions to generate increasingly harmful oxidants that promote the dysfunction of physiological processes and cellular damage [3–5]. ROS can be produced by endogenous mechanisms including oxidative phosphorylation, P450 metabolism, action of peroxisomes and activation of inflammatory cells [6,7]. ROS can be also be produced by exogenous mechanisms; in this case, ROS are generated directly or indirectly by environmental agents such as genotoxic and non-genotoxic carcinogens. Damage due to oxidative stress has been observed in cases of exposure to xenobiotics with diverse structures, including drugs and hormones. Chlorinated substances, radiation, metal ions, barbiturates, phorbol esters, diethylnitrosamine (DEN) and some peroxisome-proliferating compounds have been shown to induce oxidative stress and cell injury *in vitro* and *in vivo* [8].

Oxidative stress is a biochemical condition that occurs when intracellular antioxidants are unable to neutralize the pro-oxidants such as ROS. Under normal physiological conditions, cells possess an

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adequate antioxidant defense system to neutralize ROS. The major endogenous antioxidant enzyme system involves superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) [9]. Some non-enzymatic antioxidants are vitamins E, C and A, β-carotene, cysteine and glutathione. Glutathione is the most abundant low-molecular-mass antioxidant and it is present in a reduced form (GSH) and an oxidized form (GSSG) [2]. GSH has multiple functions, including modulation of thiol-dependent cysteine-containing enzymes and inhibition of membrane lipid peroxidation. It is a co-factor for the antioxidant enzyme glutathione peroxidase and it participates in the synthesis of proteins, nucleic acids and leukotrienes, as well as in the detoxification of xenobiotics [10,11]. In addition, the GSH/GSSG ratio serves as an indicator of changes in intracellular reduction and oxidation reactions [12]. Thus, cells are protected by an array of antioxidants that maintain a balance between pro-oxidant production and antioxidant capacity.

Oxidative stress contributes to the development of carcinogenesis by several mechanisms including DNA, lipid and protein damage, changes in intracellular signalling pathways and even changes in gene expression. Together, these oxidative modifications promote abnormal cell growth and carcinogenesis [8,13]. DNA damage includes the formation of 8-hydroxydeoxyguanosine (8-OHdG), which is the most studied DNA oxygen adduct implicated in carcinogenesis [14]. Lipid peroxidation may result in several sequelae, including structural and functional membrane modifications, protein oxidation and generation of oxidation products. These oxidation products include acrolein, crotonaldehyde, malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE); all of these are considered strong carcinogens [15,16]. Proteins are the major targets of ROS, which can alter enzyme activity, membrane transporter function and ligand-receptor interactions [7].

Induction of high ROS levels alters the antioxidant defense system by producing a feedback loop in which damage accumulation leads to the initiation, promotion and progression of carcinogenesis. Chemical hepatocarcinogenesis animal models are useful tools for studying the relationship between ROS induction and carcinogenesis. For this reason, we used in the present study one of the most accepted and widely used experimental models, the Semple-Roberts hepatocarcinogenesis model (resistant hepatocyte modified model). This model is based on the induction of altered hepatic foci by DEN, 2-acetylaminofluorene (2-AAF) and partial hepatectomy (PH) [17]. It has been used successfully to demonstrate the impact of ROS on carcinogenesis [18,19]. For its part, DEN has been shown to act as a potent carcinogen and mutagen whose metabolism generates ROS [20,21]. In the present study, we used the resistant hepatocyte

modified model to test the chemopreventive effect of quercetin, an antioxidant flavonoid, in pre-neoplastic lesion development. We found that quercetin has a differential chemopreventive effect. Our results indicate that, by modulating ROS levels and increasing the activity of antioxidant enzymes and antioxidant compounds, quercetin strengthens the endogenous antioxidant defense system, thereby conferring protection from chemical carcinogens.

Methods

Animals

Male Fischer 344 rats (180–200 g) were obtained from the Production Unit of Experimental Laboratory Animals at UPEAL-Cinvestav (México D.F., Mexico). The rats had free access to water and laboratory diet. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Chemicals and reagents

The following materials were purchased from Sigma Chemical Co. (St Louis, MO): diethylnitrosamine (DEN), 2-acetylaminofluorene (2-AAF), quercetin, the Catalase Assay Kit (Cat. No. 100) and Glutathione S-Transferase Assay kit (Cat. No. CS0410). The Lowry assay kit for protein concentration determination was purchased from Biorad (Richmond, CA). The Glutathione Peroxidase Assay Kit (Cat. No. 7512-100K) was purchased from TREVIGEN (Gaitherburg, MD).

Treatments

We used an aqueous solution containing 0.5% carboxymethylcellulose (CMC) as the vehicle for quercetin and H_2O for DEN. Groups of rats received either vehicle or quercetin 2 h before initiation of hepatocarcinoma. The group without treatment (WT) was the negative control. Protocol A consisted of a complete treatment (CT) group and a group for each of the quercetin doses of 2, 5, 10, 25 or 50 mg/kg administrated 2 h before complete treatment (Q+ CT). Groups were subjected to the procedure of the resistant hepatocyte modified model [17] (Figure 1A). Rats were initiated with 200 mg/kg DEN dissolved in H_2O, delivered intraperitoneally. At 7, 8 and 9 days after initiation, 20 mg/kg 2-AAF was administered orally to the rats. On day 10 after initiation, rats were subjected to partial hepatectomy and groups were sacrificed on day 24. In protocol B, the DEN group (DEN) was subjected to DEN treatment only and the quercetin plus DEN group (Q+DEN) was subjected to treatment with DEN plus 10 mg/kg of quercetin 2 h before initiation. In addition, a control group was evaluated with quercetin treatment 2 h before being given water instead of

Group

A   **CT**

B   **DEN**
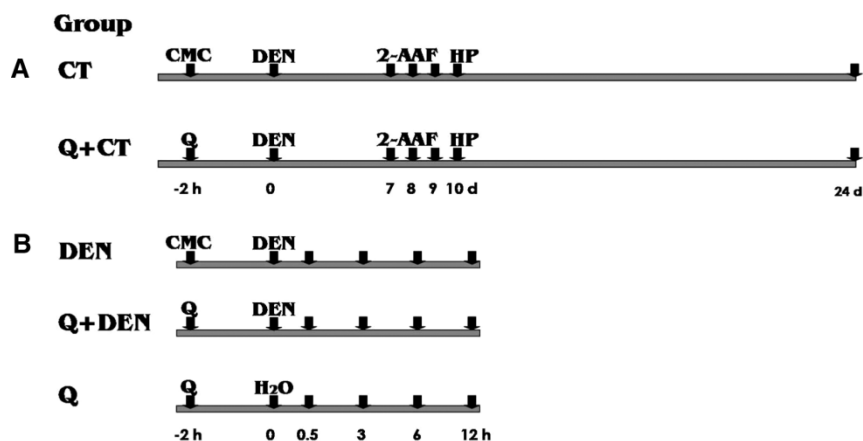
   Q+DEN

   Q

Figure 1.   Experimental design. (A) Complete treatment (CT) and quercetin plus CT (Q+CT) groups were subjected to a modified resistant hepatocyte modified model with CMC or quercetin (at doses of 2, 5, 10, 25 or 50 mg/kg) at 2 h before initiation, respectively, and sacrificed 24 days after initiation. (B) DEN and Q+DEN groups were given CMC or 10 mg/kg quercetin 2 h before initiation, respectively. Quercetin treatment (Q) was a control without DEN administration. The rats were sacrificed at 0.5, 3, 6 and 12 h after DEN administration.

DEN (Q). Finally, rats were sacrificed at 0.5, 3, 6 and 12 h after initiation (Figure 1B). Livers were removed under ether anaesthesia, washed in physiological saline solution, quickly frozen in 2-methyl butane with liquid nitrogen and stored at $-80°C$ until analysis or immersed in 10% buffered formalin for paraffin block embedding.

Histology

Sections with a thickness of 20 μm thick were prepared from the liver slices and stained to detect γ-glutamyl transpectidase (GGT) activity according to Rutenburg et al. [22]. The stained liver images were captured and GGT-positive marks were quantified using analysis software (AnalySIS Soft Imaging System GmbH, Germany).

Determination of thiobarbituric acid reactive substances (TBARS)

Samples of frozen liver were homogenized in a buffer containing 10 mmol/l Tris, phenylmethanesulphonylfluoride (PMSF) and NaCl in order to determine protein concentration. Lipid peroxidation was measured in terms of the widely used TBARS method [23]. Briefly, 650 μg of protein liver homogenates and 300 μl of 0.4% thiobarbituric acid in 20% acetic acid (pH 3.0) were mixed and heated at $100°C$ for 45 min. The samples were then cooled, 200 μl of 1.2% KCl and 0.5 ml of 1:15 pyridine/butanol were added and the samples were centrifuged at 6300 g for 10 min. The absorbance of the supernatant was measured at 532 nm. The data were interpolated from a standard curve of tetrametoxypropane (TMP), which was used as a reference control (one mol of TMP produces one mol of MDA). TBARS were expressed as nmol of MDA per mg protein.

Determination of reduced and oxidized glutathione

Liver samples were homogenized in a solution consisting of 3.75 ml of phosphate-EDTA buffer and 1 ml of 25% (v/v) H_3PO_4 and they were centrifuged at $4°C$ at 100 000 g for 20 min. To determine the amount of GSH, we mixed 0.5 ml of supernatant with 4.5 ml of phosphate-EDTA buffer (pH 8.0). The final assay mixture (2.0 ml) contained 100 μl of the diluted tissue supernatant, 1.8 ml of phosphate-EDTA and 100 μl of the O-phthaldialdehyde (OPT) solution in reagent-grade absolute methanol. This mixture was incubated at room temperature for 15 min. Fluorescence at 420 nm was determined by excitation at 350 nm. In the GSSG assay, 0.5 ml of supernatant was incubated at room temperature with 200 μl of 0.004 m N-ethylmaleimide (NEM) for 20 min to react with the GSH present in the sample. Soon after, 4.3 ml of 0.1 N NaOH were added. An aliquot (100 μl) of this mixture was taken for measurement as outlined above for the GSH assay, but the diluent was 0.1 N NaOH rather than phosphate-EDTA buffer.

Enzyme activity assays

Liver tissues were homogenized in saline solution at a ratio of 1:5 at $2°C$, protein concentration was measured and superoxide dismutase activity was determined using the xanthine/xanthine-oxidase method, which takes spectrophotometric measurements at 550 nm at room temperature [24]. The activity of glutathione peroxidase, catalase and glutathione S-transferase (GST) in liver tissue was determined according to the manufacturers' protocols for the respective kits. GPx activity was detected by using the TREVIGEN Kit. Briefly, 50 mg of liver was homogenized in 1x assay buffer (Cat. No. 7512-100-02) and readings

were taken at 340 nm. Homogenization in order to assay of CAT and GST activities was performed using the methods of Saicic et al. [25] and Xia et al. [26], with minor modifications. After this homogenization, CAT and GST activities were measured according to the procedures given by Sigma Chemical Co. Catalase assay method is based on the measurement of the hydrogen peroxide substrate remaining after catalase activity; the colourimetric method uses a substituted phenol to give a red quinoneimine dye that absorbs at 520 nm/min. Enzyme activity was expressed in units/mg of protein.

Statistical analysis

Data are expressed as the mean $\pm$ standard error (SE). All experiments were carried out on at least five animals per treatment group. Statistical significance between groups was determined by Student's t-test and $p < 0.05$ was considered statistically significant.

Results

Quercetin prevents the development of pre-neoplastic lesions

The chemopreventive effect of quercetin on the progression of pre-neoplastic lesions was evaluated using GGT detection as a tumour marker. Quercetin was administered at doses of 2, 5, 10, 25 or 50 mg/kg at 2 h before treatment with carcinogen. Quercetin at 10 mg/kg had the greatest effect on reducing total GGT-positive area, with a decrease of 70.3% relative to the CT group ($p < 0.02$) (Figure 2A). Compared with the
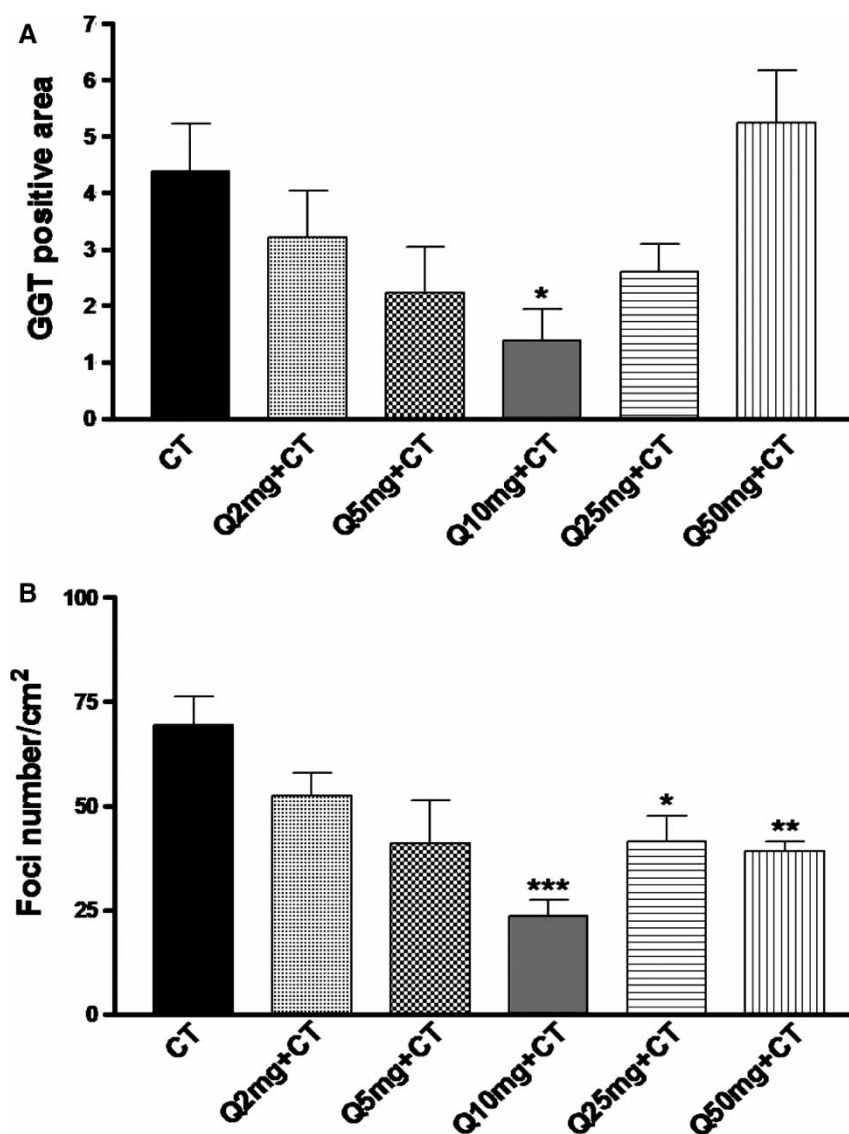

Figure 2. Effect of quercetin on GGT tumour marker activity. (A) Total GGT-positive area. (B) Number/cm^2 of GGT-positive liver lesions. A 10 mg/kg dose of quercetin showed the greatest inhibition of preneoplastic lesions. ■ CT group; ▒ Q+CT group (2 mg/kg); ▓ Q+CT group (5 mg/kg) ▒ Q+CT group (10 mg/kg) ≡ Q+CT group (25 mg/kg) ‖‖ Q+CT group (50 mg/kg). *** $p < 0.001$, ** $p < 0.03$ and * $p < 0.05$ (CT vs Q+DEN); $n = 5$ for all groups.

CT group, a quercetin dose of 10 mg/kg reduced the number of GGT-positive liver foci by 66.2% ($p < 0.001$) (Figure 2B). In addition, the changes in total GGT-positive area and foci number in the groups treated with lower doses of quercetin indicated reduced total GGT expression, although these decreases were not statistically significant. These results support the contention that, depending on dosage, quercetin can exert different effects on the development of preneoplastic lesions. The results show the chemopreventive effect of quercetin and also show that a single dose of 10 mg/kg has the greatest inhibitory effect on foci induction and, therefore, this dose was used in subsequent experiments.

Quercetin reduces DEN-induced lipid peroxidation

Lipid peroxidation is one of the major mechanisms of cellular injury caused by free radicals [27]. Because lipid peroxidation is one of the first events induced by DEN, we evaluated this process in the DEN and Q+ DEN groups at 0.5, 3, 6 and 12 h after initiation of hepatocellular carcinoma. The DEN group showed a gradual increase in the level of lipid peroxidation compared to WT group ($p < 0.001$); the levels in DEN group reached 35.2% and 169.9% above WT values at 6 and 12 h, respectively. Likewise, the Q+ DEN group increased by only 20% and 62% at 6 and 12 h ($p < 0.05$), respectively, compared to the WT group (Figure 3). However, in the Q+DEN group, lipid peroxidation was counterbalanced, so that the level was 40% lower than of the DEN group at 12 h ($p < 0.0001$). This result shows that administering quercetin 2 h before DEN drastically reduces lipid peroxidation.

Glutathione modulation by quercetin. Glutathione, the most abundant thiol antioxidant molecule, is important in the overall cellular redox balance. We found that the DEN group showed no significant variation in the reduced glutathione level compared to the WT group. In addition, the reduced glutathione level in the Q+DEN group increased by 22% at 12 h, though it decreased by 13% and 17% at 0.5 h with respect to the WT and DEN groups, respectively ($p < 0.01$) (Figure 4A). The oxidized glutathione level in the DEN group increased by 26% at 12 h ($p < 0.02$), while this level in the Q+DEN group did not show any significant variation, even though we observed a slight increase above the WT group at 12 h (Figure 4B). The GSH/GSSG ratio did not vary significantly in the DEN group with respect to the WT group. Moreover, this ratio increased in the Q+DEN group by 22% at 12 h ($p < 0.05$) compared to the WT group and by 28% at 12 h ($p < 0.01$) with respect to the DEN group (Figure 4C). Finally, the levels of total glutathione (GSH+GSSG) increased with respect to the WT group by 24.5% and 31.7% at 12 h in the DEN and Q+DEN groups, respectively, suggesting *de novo* synthesis of GSH (Figure 4D). Thus, pretreatment with quercetin restores the levels of reduced glutathione, thereby promoting homeostatic redox control.

Enzymatic activities.

Superoxide dismutase activity. One of the most effective intracellular enzymatic antioxidants is superoxide dismutase [27]. The DEN group showed a significant change in SOD activity only at 12 h, where it was 73% higher than in the WT group ($p < 0.01$) (Figure 5A). In contrast, SOD activity in the Q+DEN group increased early and continuously from 0.5 h until 12 h; the activity was, respectively, 72%, 60%, 84% and 89% higher than in the WT group ($p < 0.01$). The SOD activity in the Q+DEN group was higher than that in the DEN group by 75%, 140% and 98% at 0.5, 3 and 6 h, respectively ($p < 0.01$). SOD activity
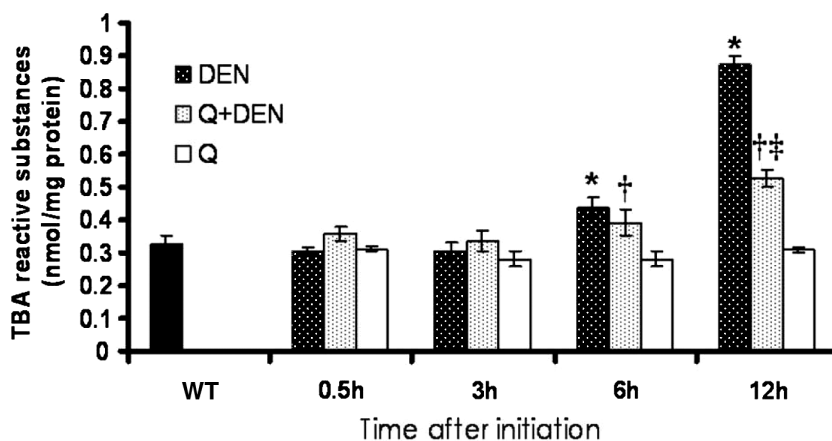

Figure 3.   Quercetin inhibits lipid peroxidation induced by DEN. Lipid peroxidation was determined in terms of TBARS at 0.5, 3, 6 and 12 h post-initiation. Quercetin reduced the lipid peroxidation level by 40% at 12 h with respect to the DEN group. ■ WT group; ▨ DEN group; ▨ Q+DEN group; □ Q group. ⋆ $p < 0.001$ (WT vs DEN), † $p < 0.05$ (WT vs Q+DEN) and ‡ $p < 0.0001$ (DEN vs Q+DEN); $n = 5$ for all groups.
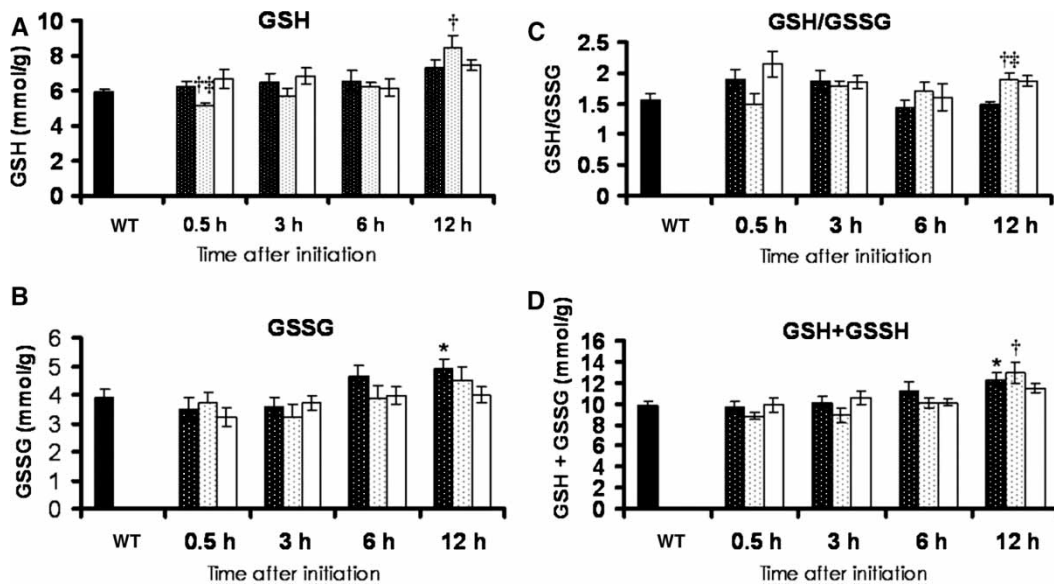
Figure 4. Reduced (GSH) and oxidized (GSSG) glutathione, GSH/GSSG ratio and total glutathione (GSH+GSSG). (A) GSH. The Q+DEN group showed a GSH level 22% higher than that of the WT group at 12 h. (B) GSSG. The DEN group showed GSSG levels 26% higher than those of the WT group at 12 h. (C) GSH/GSSG ratio. The Q+DEN group increased the GSH/GSSG ratio at 12 h. (D) GSH+GSSG. In DEN and Q+DEN groups, total GSH/GSSG was increased at 12 h. ■ WT group; ▓ DEN group; ▨ Q+DEN group; □ Q group. $\star p < 0.02$ (WT vs DEN), $\dagger p < 0.05$ (WT vs Q+DEN) and $\ddagger p < 0.03$ (DEN vs Q+DEN); $n = 5$ in all groups.

increased in the presence of quercetin as early as 0.5 h after initiation of hepatocarcinoma, indicating that pre-induced SOD activity can prevent DEN-induced accumulation of superoxide and lipid peroxidation.

Catalase activity. Catalase has the highest turnover rate of all antioxidant enzymes: one molecule of catalase can convert ~ 6 million molecules of H_2O_2 to H_2O and O_2 every minute [14]. Catalase activity in
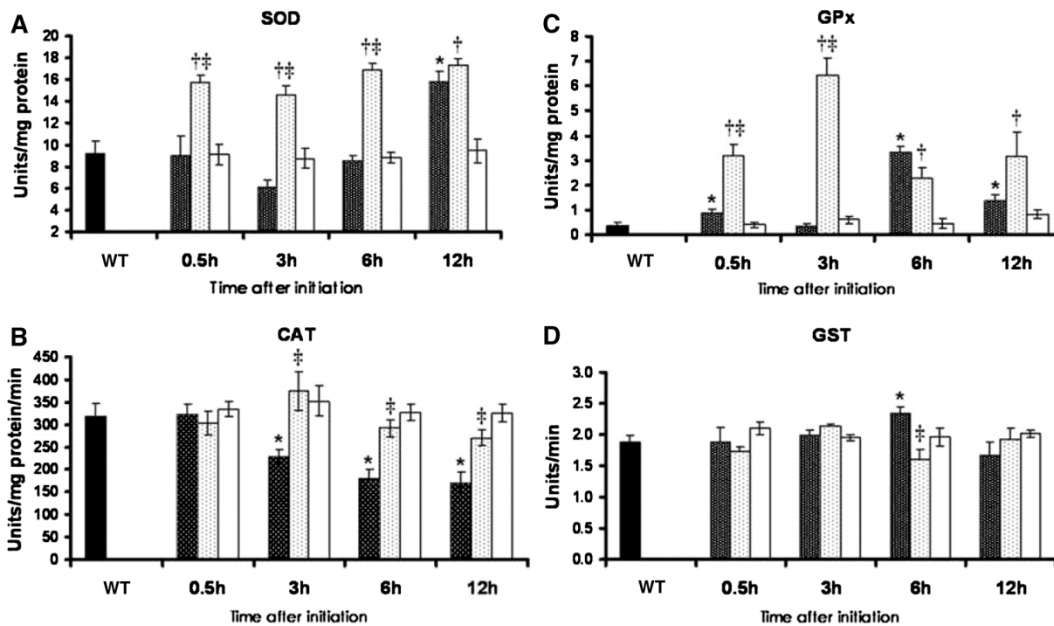

Figure 5. Effects of DEN and quercetin pre-treatment on enzymatic activities. (A) Effects of DEN and quercetin pre-treatment on SOD activity in rat liver. SOD activity increased in the DEN group by 73% until 12 h and by 72%, 60%, 84% and 89% in the Q+DEN group at 0.5, 3, 6 and 12 h, respectively, with respect to the WT group. (B) Effect of DEN and quercetin pre-treatment on CAT activity in rat liver. CAT activity was decreased in the DEN group at 3, 6 and 12 h with respect to the WT group. (C) Effect of DEN and quercetin pre-treatment on glutathione peroxidase (GPx) activity in rat liver. The DEN and Q+DEN group showed increases in GPx activity relative to the WT group. (D) Effect of DEN and quercetin pre-treatment on glutathione S-transferase (GST) activity in rat liver. GST activity was changes in DEN and Q+DEN groups at 6 h with respect to the WT group. ■ WT group; ▓ DEN group; ▨ Q+DEN group; □ Q group. $\star p < 0.03$ (WT vs DEN), $\dagger p < 0.01$ (WT vs Q+DEN) and $\ddagger p < 0.01$ (DEN vs Q+DEN); $n = 5$ for all groups.

the DEN group was lower than in the WT group by 27%, 43% and 48% at 3 h, 6 h and 12 h post-initiation ($p \leq 0.03$) (Figure 5B). The Q + DEN group showed the same catalase activity as the WT group. Nevertheless, CAT activity in the Q + DEN group was 64%, 62% and 62% higher than in the DEN group at 3, 6 and 12 h post-initiation, respectively ($p \leq 0.01$). Thus, quercetin maintains normal levels of catalase activity. The results with catalase activity are similar to the results with SOD activity, which suggests that quercetin increases both activities and thereby strengthens their antioxidant effect.

Glutathione peroxidase activity. Glutathione peroxidase is another enzyme capable of reducing hydroperoxides, including lipid hydroperoxides, and it does so by using two glutathione molecules (GSH) as substrates [11,12]. In the DEN group, GPx activity increased to 147%, 821% and 282.3% above levels in the WT group at 0.5, 6 and 12 h post-initiation, respectively ($p < 0.03$) (Figure 5C). However, they were smaller than the differences in GPx activity between the Q + DEN and WT groups, being 789.8%, 1,694.1%, 532.3% and 782.3% at 0.5, 3, 6 and 12 h respectively ($p < 0.01$). The GPx activity was higher in the Q + DEN group with respect to the DEN group, which was 260% and 1933% at 0.5 and 3 h post-initiation, respectively ($p < 0.001$). These results clearly show that quercetin increases the activity of the three antioxidant enzymes in this study. The activity of GPx increased the most and the early increase in GPx activity in the Q + DEN group may prevent DEN-induced lipid peroxidation.

Glutathione S-transferase activity. Glutathione S-transferase catalyses the conjugation of glutathione to electrophiles. We found that GST activity in the DEN group increased by only 24.6% at 6 h compared to the WT group. In the Q + DEN group, GST activity fell by 31.3% compared to the DEN group at 6 h post-initiation ($p \leq 0.01$) (Figure 5D). Thus, a differential GST activity response was detected 6 h after initiation of hepatocarcinoma.

Effect of quercetin treatment alone

Surprisingly, when quercetin was administered to the non-DEN treated rats, no significant changes were observed in any of the enzyme activities assayed. Similarly, there were no significant changes in the values for oxidized, reduced or total glutathione or in the ratio between oxidized and reduced glutathione.

Discussion

In the present study, we showed that quercetin exerts a chemopreventive effect by inhibiting lipid peroxidation increasing levels of GSH and total glutathione,

raising the GSH/GSSG ratio and boosting the activity of SOD, CAT and GPx. Epidemiologic studies have shown that quercetin protects against various stages in carcinogenesis; nevertheless, the precise mechanism of this protection has been unclear [28]. Previous studies have shown quercetin to have a dual effect. A low dose of quercetin protects H4IIE rat hepatoma cells against H_2O_2 cytotoxicity, while higher doses induce DNA damage [28]. In different experimental models, the actions of natural and synthetic chemical agents have been proven to inhibit carcinogenesis. In this context, antioxidant agents have been shown to possess significant chemopreventive effects and many experimental attempts have been made to address their mechanisms of action [29,30]. Curcumin, the yellow pigment isolated from the rhizomes of *Curcuma longa* Linn, inhibits chemically-induced carcinogenesis at multiple organ sites in various experimental models [31,32]. The compound was found to inhibit inflammation, hyperplasia, proliferation, generation of ROS and oxidative DNA damage in mouse skin [31,33]. Resveratrol, a phytoalexin present in grapes and other plants, exerts antioxidant, anti-inflammatory and chemopreventive activities by modulating diverse events in cellular signalling [34]. Each antioxidant chemopreventive agent works by a different mechanism of action, although they share the ability to inhibit the negative effects of oxidative stress during carcinogenesis.

Here, we show that quercetin counteracts lipid peroxidation and prevents the appearance of pre-neoplastic lesions that are usually induced by carcinogen treatment in the resistant hepatocyte modified model [18]. Previous studies have shown quercetin has a chemopreventive effect that involves inhibiting lipid peroxidation. Nevertheless, there are no published studies investigating the relation to the antioxidant defense system. Therefore, the aim of this study was to analyse the anti-carcinogenic effect of quercetin and its effects on ROS and the antioxidant defense system during initiation of chemical carcinogenesis. First, we confirmed the chemoprotective effect of quercetin in studies that defined 24 days as the end point of pre-neoplastic lesions induction. We found that a single administration of different quercetin concentrations induces the hormesis phenomenon, with the optimal chemopreventive dose being 10 mg/kg given 2 h before initiation of hepatocarcinoma. Our chemopreventive results are in agreement with another *in vitro* study, which found that 50 mmol/L of quercetin suppresses proliferation in several different human hepatocellular carcinoma lines. Our results are also consistent with an *in vivo* report showing that a 2% solution of dietary quercetin inhibits intestinal hyperproliferation and focal dysplasia induced by azoxymethane in mice [35–38]. This intestinal carcinogen produces DNA alkylation, which is similar to the alkylation produced by

DEN, and this phenomenon has been postulated to be the alteration responsible for cancer induction. Nevertheless, we contend that oxidative stress is necessary to induce cell proliferation in order to establish a permanent phenotypic change [18,39,40].

Lipid peroxidation is a useful marker of oxidative stress because it is linked to the increased production of ROS caused when cytochrome P450 enzymes metabolize DEN [37]. We show here that quercetin pre-treatment efficiently reduces DEN lipid peroxidation levels, suggesting that counteracting this oxidative step is decisive in avoiding the development of pre-neoplastic lesions. The products of lipid peroxidation are considered mutagenic and carcinogenic and they cause damage to cellular macromolecules by generating ROS [37,41]. In agreement with our study, previous studies of quercetin have reported that this flavonoid protects against cytotoxicity caused by hydrogen peroxide and ethanol [28,38]. Similarly, *in vivo* studies of cervix uteri carcinogenesis in mice found that quercetin administration as a 2% component in the diet inhibited lipid peroxidation [42].

In this study, we found that quercetin treatment affected all of the components of the antioxidant defense system analysed in the same 'direction', namely towards counteracting oxidative stress. Glutathione is a very important factor in this system: although DEN treatment did not significantly change the level of GSH, quercetin increased the level of glutathione. These results indicate that quercetin creates an unfavourable environment for carcinogenesis. We suggest that the increased GSH level caused by quercetin is related to the observed decrease in lipid peroxidation. Total GSSG levels are low compared with the high total level of GSH and a minor elevation in the oxidation of GSH to GSSG results in a significant elevation in the intracellular level of GSSG [12,43]. Furthermore, the GSH/GSSG ratio, which is often used as an indicator of the cellular redox state [11,44], significantly increased only in the quercetin-pre-treated group. Congruent with this result, the quercetin-pre-treated group also showed an elevated total glutathione level (GSH + GSSG) at 12 h post-initiation with respect to the group without. Taken together, these results suggest that quercetin treatment produces an antioxidant state that favours homeostatic redox control.

Only the DEN group showed an increase in GSSG levels, reflecting an escalation in the oxidative stress that correlates closely with an increase in lipid peroxidation. Even in the presence of oxidative stress, the physiological concentrations of GSH and GSSG are maintained via an induction of GSH synthesis or elimination of GSSG through increased glutathione reductase activity [7]. This suggests that quercetin activates *de novo* synthesis of glutathione. In support of this idea, quercetin has been reported to induce a transcription factor complex in human GepG2 cells that targets the antioxidant-responsive element/electrophile-responsive element (ARE/EpRE). This increases ARE binding activity and Nrf2-mediated transcription activity [45]. Nrf2, a member of the CNC family of bZIP proteins, up-regulates various antioxidant enzymes, including those participating in GSH synthesis [9,11]. It is tempting to propose that the increase in total glutathione, also observed 12 h after DEN treatment, is related to the lipid peroxidation increase through the formation of HNE, which regulates GSH biosynthesis by the c-Jun N-terminal kinase (JNK) pathway [14]. In the present study, quercetin was found to reduce the development of pre-neoplastic lesions and lipid peroxidation levels. In addition, the increase in GSH, the GSH/GSSG ratio and total glutathione suggests a direct antioxidant effect.

In addition to these effects on antioxidant molecules such as glutathione, quercetin increases the activity of antioxidant enzymes. GPx is an antioxidant enzyme participating in the defense mechanism that is activated before the initiation of chronic oxidative stress. The moderate changes observed in the activity of liver GPx in the DEN group may be a late-stage protective response to peroxide generation induced by DEN. These moderate changes contrast with the exponential changes in enzyme levels observed in the quercetin-pre-treated group at 0.5 and 3 h after initiation of hepatocarcinoma. Even when these levels decreased after 6 h and at 12 h post-initiation, GPx activity remained several-fold higher compared to the group without treatment. GPx activity correlated with the levels of lipid peroxidation observed in the quercetin pre-treatment group, suggesting that DEN induces a chronic oxidative state.

SOD acts as the first line of defense against superoxide radicals, which are able to dismutate two superoxide radical to H_2O_2 and O_2. In addition, CAT and GPx act as supporting antioxidant enzymes by converting H_2O_2 to H_2O, thereby providing protection against ROS. The reduction in activity of these antioxidant enzymes may be caused by the increase in radical production during DEN metabolism. DEN administration decreases CAT activity in liver tissues by $\sim 50\%$. As shown in other experimental models, quercetin induction of GPx and SOD prevents the accumulation of ROS and DNA damage [42]. The increase in GST activity in the liver after exposure to DEN at 6 h suggests that GSH may participate in the conjugation reactions of toxic substances.

Finally, *in vitro* and *in vivo* cancer models have shown that quercetin exerts a differential effect depending of the experimental conditions [36,46,47]. According at our results, in animals not treated with carcinogens quercetin did not modify enzymatic and

non-enzymatic antioxidant defense systems. Quercetin can be adsorbed and excreted with no accumulation in tissues and biological fluids [46]. Under normal conditions the quercetin molecule could be excreted without biotransformation without exerting its properties. Otherwise, under homeostatic alteration induced by carcinogens, quercetin could act by as a chemopreventive agent. DEN administration bears a strong cellular damage during its metabolism and increases accumulation of ROS. When quercetin is administrated in these conditions, it is oxidized. We can speculate that this modification gives quercetin the ability to bioactivate the antioxidant defense system and this also has been proposed by other authors [40,48,49].

In summary, quercetin increases or stabilizes all of the components of the antioxidant defense system that were examined in this study: GSH, GSH/GSSG, GSH + GSSG, SOD, CAT and GPx. These factors protect cells from ROS damage in DEN-induced hepatocellular carcinogenesis, as seen in the ability of quercetin to decrease lipid peroxidation. We propose that quercetin abolishes the oxidative state induced by the initiator, DEN, and that it does so not by interacting directly with ROS, but by activating the antioxidant defense system. We conclude that quercetin counteracts the lipid peroxidation induced by increased ROS generation and thereby prevents the appearance of pre-neoplastic lesions. These findings support the conclusion that oxidative stress is a necessary step in the production of pre-neoplastic lesions in a hepatocellular carcinoma model.

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References

[1] Ozben T. Oxidative stress and apoptosis: impact on cancer therapy. J Pharm Sci 2007;96:2181–2196.

[2] Karihtala P, Soini Y. Reactive oxygen species and antioxidant mechanisms in human tissues and their relation to malignancies. Apmis 2007;115:81–103.

[3] Benhar M, Engelberg D, Levitzki A. ROS, stress-activated kinases and stress signaling in cancer. EMBO Rep 2002;3:420–425.

[4] Haddad JJ. Antioxidant and prooxidant mechanisms in the regulation of redox(y)-sensitive transcription factors. Cell Signal 2002;14:879–897.

[5] Das U. A radical approach to cancer. Med Sci Monit 2002;8:RA79–RA92.

[6] Waris G, Ahsan H. Reactive oxygen species: role in the development of cancer and various chronic conditions. J Carcinog 2006;5:14.

[7] Klaunig JE, Kamendulis LM. The role of oxidative stress in carcinogenesis. Annu Rev Pharmacol Toxicol 2004;44:239–267.

[8] Klaunig JE, Xu Y, Isenberg JS, Bachowski S, Kolaja KL, Jiang J, Stevenson DE, Walborg EF Jr. The role of oxidative stress in chemical carcinogenesis. Environ Health Perspect 1998;106(Suppl 1):289–295.

[9] Surh YJ, Kundu JK, Na HK, Lee JS. Redox-sensitive transcription factors as prime targets for chemoprevention with anti-inflammatory and antioxidative phytochemicals. J Nutr 2005;135:2993S–3001S.

[10] Kim HG, Hong SM, Kim SJ, Park HJ, Jung HI, Lee YY, Moon JS, Lim HW, Park EH, Lim CJ. Age-related changes in the activity of antioxidant and redox enzymes in rats. Mol Cells 2003;16:278–284.

[11] Wu G, Fang YZ, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. J Nutr 2004;134:489–492.

[12] Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med 2001;30:1191–1212.

[13] Cerutti P, Ghosh R, Oya Y, Amstad P. The role of the cellular antioxidant defense in oxidant carcinogenesis. Environ Health Perspect 1994;102(Suppl 10):123–129.

[14] Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 2006;160:1–40.

[15] Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007;39:44–84.

[16] Bartsch H, Nair J. Chronic inflammation and oxidative stress in the genesis and perpetuation of cancer: role of lipid peroxidation, DNA damage, and repair. Langenbecks Arch Surg 2006;391:499–510.

[17] Semple-Roberts E, Hayes MA, Armstrong D, Becker RA, Racz WJ, Farber E. Alternative methods of selecting rat hepatocellular nodules resistant to 2-acetylaminofluorene. Int J Cancer 1987;40:643–645.

[18] Sanchez-Perez Y, Carrasco-Legleu C, Garcia-Cuellar C, Perez-Carreon J, Hernandez-Garcia S, Salcido-Neyoy M, Aleman-Lazarini L, Villa-Trevino S. Oxidative stress in carcinogenesis. Correlation between lipid peroxidation and induction of preneoplastic lesions in rat hepatocarcinogenesis. Cancer Lett 2005;217:25–32.

[19] Carrasco-Legleu CE, Sanchez-Perez Y, Marquez-Rosado L, Fattel-Fazenda S, Arce-Popoca E, Hernandez-Garcia S, Villa-Trevino S. A single dose of caffeic acid phenethyl ester prevents initiation in a medium-term rat hepatocarcinogenesis model. World J Gastroenterol 2006;12:6779–6785.

[20] Mittal G, Brar AP, Soni G. Impact of hypercholesterolemia on toxicity of N-nitrosodiethylamine: biochemical and histopathological effects. Pharmacol Rep 2006;58:413–419.

[21] Verna L, Whysner J, Williams GM. N-nitrosodiethylamine mechanistic data and risk assessment: bioactivation, DNA-adduct formation, mutagenicity, and tumor initiation. Pharmacol Ther 1996;71:57–81.

[22] Rutenburg AM, Kim H, Fischbein JW, Hanker JS, Wasserkrug HL, Seligman AM. Histochemical and ultrastructural demonstration of gamma-glutamyl transpeptidase activity. J Histochem Cytochem 1969;17:517–526.

[23] Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol 1978;52:302–310.

[24] McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 1969;244:6049–6055.

[25] Saicic ZS, Mijalkovic DN, Nikolic AL, Blagojevic DP, Spasic MB. Effect of thyroxine on antioxidant defense system in the liver of different aged rats. Physiol Res 2006;55:561–568.

[26] Xia E, Rao G, Van Remmen H, Heydari AR, Richardson A. Activities of antioxidant enzymes in various tissues of male Fischer 344 rats are altered by food restriction. J Nutr 1995;125:195–201.

[27] Popov B, Gadjeva V, Valkanov P, Popova S, Tolekova A. Lipid peroxidation, superoxide dismutase and catalase activities in brain tumor tissues. Arch Physiol Biochem 2003;111:455–459.

[28] Watjen W, Michels G, Steffan B, Niering P, Chovolou Y, Kampkotter A, Tran-Thi QH, Proksch P, Kahl R. Low concentrations of flavonoids are protective in rat H4IIE cells whereas high concentrations cause DNA damage and apoptosis. J Nutr 2005;135:525–531.

[29] Pauwels EK, Erba PA, Kostkiewicz M. Antioxidants: a tale of two stories. Drug News Perspect 2007;20:579–585.

[30] Lee SB, Cha KH, Selenge D, Solongo A, Nho CW. The chemopreventive effect of taxifolin is exerted through ARE-dependent gene regulation. Biol Pharm Bull 2007;30:1074–1079.

[31] Huang MT, Newmark HL, Frenkel K. Inhibitory effects of curcumin on tumorigenesis in mice. J Cell Biochem Suppl 1997;27:26–34.

[32] Li N, Chen X, Liao J, Yang G, Wang S, Josephson Y, Han C, Chen J, Huang MT, Yang CS. Inhibition of 7,12-dimethyl-benz[a]anthracene (DMBA)-induced oral carcinogenesis in hamsters by tea and curcumin. Carcinogenesis 2002;23:1307–1313.

[33] Conney AH, Lu Y, Lou Y, Xie J, Huang M. Inhibitory effect of green and black tea on tumor growth. Proc Soc Exp Biol Med 1999;220:229–233.

[34] Aggarwal BB, Bhardwaj A, Aggarwal RS, Seeram NP, Shishodia S, Takada Y. Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. Anticancer Res 2004;24:2783–2840.

[35] Shi M, Wang FS, Wu ZZ. Synergetic anticancer effect of combined quercetin and recombinant adenoviral vector expressing human wild-type p53, GM-CSF and B7-1 genes on hepatocellular carcinoma cells *in vitro*. World J Gastroenterol 2003;9:73–78.

[36] Williamson G, Manach C. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. Am J Clin Nutr 2005;81:243S–255S.

[37] Aiub CA, Pinto LF, Felzenszwalb I. N-Nitrosodiethylamine mutagenicity at low concentrations. Toxicol Lett 2003;145:36–45.

[38] Molina MF, Sanchez-Reus I, Iglesias I, Benedi J. Quercetin, a flavonoid antioxidant, prevents and protects against ethanol-induced oxidative stress in mouse liver. Biol Pharm Bull 2003;26:1398–1402.

[39] Yamada K, Yamamiya I, Utsumi H. *In vivo* detection of free radicals induced by diethylnitrosamine in rat liver tissue. Free Radic Biol Med 2006;40:2040–2046.

[40] Sivaramakrishnan V, Shilpa PN, Praveen Kumar VR, Niranjali Devaraj S. Attenuation of N-nitrosodiethylamine-induced hepatocellular carcinogenesis by a novel flavonol-Morin. Chem Biol Interact 2008;171:79–88.

[41] Pradeep K, Mohan CV, Gobianand K, Karthikeyan S. Effect of Cassia fistula Linn. leaf extract on diethylnitrosamine induced hepatic injury in rats. Chem Biol Interact 2007;167:12–18.

[42] De S, Chakraborty J, Chakraborty RN, Das S. Chemopreventive activity of quercetin during carcinogenesis in cervix uteri in mice. Phytother Res 2000;14:347–351.

[43] Tew KD. Redox in redux: Emergent roles for glutathione S-transferase P (GSTP) in regulation of cell signaling and S-glutathionylation. Biochem Pharmacol 2007;73:1257–1269.

[44] Hayes JD, Flanagan JU, Jowsey IR. Glutathione transferases. Annu Rev Pharmacol Toxicol 2005;45:51–88.

[45] Tanigawa S, Fujii M, Hou DX. Action of Nrf2 and Keap1 in ARE-mediated NQO1 expression by quercetin. Free Radic Biol Med 2007;42:1690–1703.

[46] Murakami A, Ashida H, Terao J. Multitargeted cancer prevention by quercetin. Cancer Lett 2008;269:315–325.

[47] Lamson DW, Brignall MS. Antioxidants and cancer, part 3: quercetin. Altern Med Rev 2000;5:196–208.

[48] Boots AW, Kubben N, Haenen GR, Bast A. Oxidized quercetin reacts with thiols rather than with ascorbate: implication for quercetin supplementation. Biochem Biophys Res Commun 2003;308:560–565.

[49] Dias AS, Porawski M, Alonso M, Marroni N, Collado PS, Gonzalez-Gallego J. Quercetin decreases oxidative stress, NF-kappaB activation, and iNOS overexpression in liver of streptozotocin-induced diabetic rats. J Nutr 2005;135:2299–2304.